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Physiological Events in *Clostridium acetobutylicum* during the Shift from Acidogenesis to Solventogenesis in Continuous Culture and Presentation of a Model for Shift Induction

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The pH of continuous cultures of *Clostridium acetobutylicum* growing at pH 5.6 was allowed to decrease to 4.3 after acid production and thereby to shift the cultures from acetate and butyrate to acetone and butanol formation. Several parameters were determined during the shift. An increase in the intracellular acid concentration to 440 mM was recorded. An excess of undissociated butyric acid but not of acetic acid just before the shift to solventogenesis was followed by a decline in acid production and subsequently by the uptake of acids. The intracellular ATP concentration reached a minimum before the onset of solventogenesis; this presumably reflects the ATP-consuming proton extrusion connected with the increase in the ΔpH from 0.7 to 1.4 units. The pool of NADH plus NADPH exhibited a drastic increase until solventogenesis was induced. The changes in the ATP and ADP and NADH plus NADPH pools during these pH shift experiments were the beginning of a stable metabolic oscillation which could also be recorded as an oscillation of the culture redox potential under steady-state solventogenic conditions. Similar changes were observed when the shift was induced by the addition of butyrate and acetate (50 mM each) to the continuous culture. However, when methyl viologen was added, important differences were found: ATP levels did not reach a minimum, acetoacetate decarboxylase activity could not be measured, and butanol but not acetone was produced. A model for the shift is proposed; it assumes the generation of two signals, one by the changed ATP and ADP levels and the other by the increased NAD(P)H level.

Clostridium acetobutylicum was of industrial interest between 1915 and 1950 because of its ability to ferment molasses or starch to the organic solvents butanol and acetone. During growth of *C. acetobutylicum* in batch culture, butyric and acetic acids are produced first; the culture then undergoes a shift, and solvents are formed. Several attempts have been made to define the shift-inducing conditions and to optimize solvent yield (4, 19, 35). As a result, several parameters affecting solventogenesis are now known.

First, decreasing the pH from 6.0 to 4.5 can be used to induce the shift both in batch and in continuous culture (2, 3). The intracellular pH decreases less under these conditions than the extracellular pH. Since undissociated organic acids tend to attain similar concentrations inside and outside, the increase in ΔpH means that there are higher butyrate and acetate concentrations inside than outside the cell (8, 13, 16, 30, 38). High intracellular acid concentrations seem to be related to the shift, since it has been observed that the shift can be induced by the addition of butyrate to high concentrations at neutral pH (15, 27) as well as by the addition of propionate, valerate, or 4-hydroxybutyrate (18). Similarly, a combination of acetoacetate and butyrate with uncouplers such as carbonylcyanide *p*-trifluoromethoxyphenylhydrazone and carbonylcyanide *m*-chlorophenylhydrazone also leads to shift induction (17), as does the addition of the nonmetabolizable acid 5,5-dimethyloxazolidine-2,4-dione (12). Other manipulations to induce solvent formation, especially of butanol, include gassing with carbon monoxide, which inhibits the hydrogenase (20, 29); adding methyl viologen (34); and increasing the partial pressure of molecular hydrogen in the culture (10).

We have studied the metabolic consequences of some of these treatments in continuous culture, and we present here a two-signal model for the shift in accordance with the known shift-inducing conditions.

MATERIALS AND METHODS

Organism and culture conditions. The experiments were carried out with *C. acetobutylicum* DSM 1731. The fermentation medium was phosphate limited and had the following composition (in grams per liter): glucose · H_2O, 40; KH_2PO_4, 0.05; $(NH_4)_2SO_4$, 2; $MgSO_4 \cdot 7H_2O$, 0.1; NaCl, 0.01; $Na_2MoO_4 \cdot 2H_2O$, 0.01; $CaCl_2 \cdot 2H_2O$, 0.01; $MnSO_4 \cdot H_2O$, 0.015; $FeSO_4 \cdot 7H_2O$, 0.015; biotin, 0.0001; thiamine hydrochloride, 0.002; and *p*-aminobenzoic acid, 0.002 in distilled water. The medium was acidified to pH 2 to keep the salts in solution and sterilized by passage through a Seitz filter EKS (14 cm) with an N_2 pressure of 3×10^5 Pa. The pH in the fermentor vessel was measured with a glass electrode (Cahn-Ingold, Frankfurt/Main, Germany) and controlled by the addition of 4 N KOH.

Shift experiments. All shift experiments were carried out in a Biostat M fermentor (Braun, Melsungen, Germany) with a working volume of 0.8 liters. The standard conditions were as follows: dilution rate, 0.08 to 0.09 h^{-1}; pH, 5.6 to 5.8; temperature, 37°C; agitation, 250 rpm; no gassing. The concentrations of the fermentation products were about 60 mM for butyrate and 30 mM for acetate; those of the solvents were below 5 mM.

The pH shift experiments were started by switching off the pH control of the fermentor and allowing the pH to drop to

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4.3. The pH control was then switched on to keep the pH at this value. At the new steady state, the concentrations of the fermentation products were ≤10 mM for butyrate, ≤18 mM for acetate, about 30 mM for butanol, and 15 to 20 mM for acetone.

In the acid shift, potassium butyrate and potassium acetate (50 mM each) were added to the continuous culture running under standard conditions. This high acid concentration was maintained by continuous pumping of neutralized acids into the fermentor vessel.

The methyl viologen shift was started by adding methyl viologen to the culture at a final concentration of 1 mM. This concentration was also held constant by pumping methyl viologen continuously into the culture.

Samples (10 ml) were withdrawn into evacuated Hungate tubes. Aliquots (1 ml) were centrifuged through 0.2 ml of silicon oil (density, 1.05 g/ml) for 2 min in a Microfuge (Beckman, Fullerton, Tex.) at 13,000 rpm. The supernatant was removed and frozen at −20°C for substrate and product determinations. The silicon oil was removed by suction, and the cell pellets were frozen in liquid nitrogen and stored at −80°C until pool concentrations were determined.

Determination of growth parameters. The redox potential of the culture was measured on-line with a redox electrode (Cahn Ingold).

The optical density was determined in a spectrophotometer (Carl Zeiss, Oberkochen, Germany) at a wavelength of 578 nm against water. Samples with an optical density of more than 0.3 were diluted with water. The determination of protein content was done as described before (23, 37).

Product analysis. Quantitative analysis of the solvents and acids was done by gas chromatography. A 1-μl amount of an acidified sample was injected into a Perkin-Elmer 3920 gas chromatograph (Perkin-Elmer, Überlingen, Germany) equipped with a flame ionization detector. The 2-m glass column was packed with Chromosorb 101 (80-100 mesh). The carrier gas was N_2, at a flow rate of 30 ml/min. The oven temperature was 160 to 170°C, the temperature of the injector was 200°C, and that of the detector 220°C. Propionic acid was used as an internal standard, and the calculations were done on a two-channel Chromatopac C-R2AX integrator with an INP-R2A interface (Shimadzu Corp., Duisburg, Germany).

Determination of pool concentrations. The internal pH and the cell volume were determined by the distribution of isotopically labeled compounds (13). Instead of [^{14}C]dextran, [^{14}C]taurine was used for internal volume measurements.

For the determination of internal acid contents, 100 μl of 0.6 M perchloric acid was pipetted onto the pellet after silicon oil centrifugation as described above. The suspension was mixed, incubated overnight at 0°C, again mixed vigorously, and centrifuged for 10 min at 4°C and 15,000 rpm (3MK; Sigma, Osterode/Harz, Germany). The supernatant was analyzed by gas chromatography as described above.

Coenzyme A (CoA) derivative contents were determined by reversed-phase high-pressure liquid chromatography (HPLC). Two pellets (prepared by silicon oil centrifugation) were suspended in 200 μl of 0.6 M perchloric acid. Dithioerythritol was added to a final concentration of 5 mM. The suspension was mixed, incubated overnight at 0°C, again mixed vigorously, and centrifuged for 10 min at 15,000 rpm and 4°C. Then, 140 μl of the supernatant was adjusted to pH 4.5 with 3.2 M KOH–0.5 M citric acid. Analysis was done on a Hypersil ODS column (250 by 4.5 mm; I. Molnar HPLC Technology, Berlin, Germany) with a Knauer HPLC, which consisted of an HPLC autosampler, HPLC programmer model 50, two HPLC pumps (model 64), a high-temperature oven which was set to 30°C, and a variable-wavelength monitor (Knauer, Bad Homburg, Germany). The separation system consisted of buffer A (0.2 M NaH_2PO_4 [pH 4.5]) and buffer B (0.2 M NaH_2PO_4 [pH 4.5], 20% [vol/vol] acetonitrile), which were mixed according to the following protocol: flow rate, 1.5 ml/min; 0 to 5 min, buffer A; 5 to 25 min, buffer B increasing up to 15%; 25 to 30 min, buffer B maintained at 15%; 30 to 35 min, buffer B increasing up to 37%; 35 to 40 min, buffer B maintained at 37%; 40 to 60 min, buffer B increasing to 100%; 60 to 65 min, buffer B; 65 to 67 min, changing buffer B to buffer A. The concentration was determined by using a calibration curve of between 0.025 and 0.1 mM for each CoA derivative at 260 nm. The experimental error was 9.8% for HS-CoA, 1.3% for acetyl-CoA, and 6.2% for butyryl-CoA.

The ATP content was measured with the luciferin-luciferase system of *Photinus pyralis* (firefly) (22). Cell suspensions (800 μl) were transferred directly from the fermentor to 200 μl of ice-cold 3 M perchloric acid. The suspension was mixed vigorously several times while incubating on ice for 45 to 60 min. The cell extract was neutralized by the addition of 160 μl of 5 M KOH–0.5 M potassium phosphate buffer (pH 7) and centrifuged for 5 min at 4°C and 13,000 rpm. From 5 to 20 μl of the sample was analyzed in a Biocounter M 1500 L (Lumac, Landgraaf, The Netherlands). The amounts were determined by using a calibration curve of between 5 and 70 pmol of ATP, with an experimental error of 3.4%.

For determination of the ADP plus ATP content of the cells, 100 μl of neutralized cell extract was mixed with 10 μl of pyruvate kinase buffer (0.1 M Tris-hydrochloride [pH 7.4], 50 mM phosphoenolpyruvate, 35 mM KCl, 6 mM $MgCl_2$, 15 U of pyruvate kinase per μl) and incubated for 90 min at room temperature. After the addition of 5 μl of ice-cold 60% perchloric acid and incubation on ice for 10 min, the cell extract was neutralized with 30 μl of 2 M KOH–50 mM potassium phosphate buffer (pH 7) and centrifuged, and the supernatant was analyzed as described above.

The cellular AMP plus ADP plus ATP content was analyzed after conversion to ATP by pyruvate kinase-myokinase reactions. Neutralized cell extract (100 μl) was mixed with 10 μl of pyruvate kinase buffer, which additionally contained 10 U of myokinase per ml, and treated as described above.

NAD(P)H. NAD(P)H was measured biolumetrically with the luciferase-flavin mononucleotide reductase of *Photobacterium fisheri* with a test system from Boehringer (Mannheim, Germany). Cell suspension (1 ml) from the fermentor was mixed directly with 100 μl of 3 M KOH, incubated for 5 min at 50°C, neutralized with 80 μl of 3 M HCl, and centrifuged for 5 min at 13,000 rpm. From 50 to 100 μl of a 10-fold-diluted cell extract was added to 300 μl of test solution and mixed, and after 15 s, the bioluminescence was measured for 10 s several times in a Biocounter M 1500 L. The highest counts per 10 s were directly proportional to the amount of NAD(P)H, which was determined by using a calibration curve of between 0.2 and 2 pmol, with an experimental error of 5.6%.

On-line measurements of NAD(P)H were done with a fluorescence detector (RF 530; Shimadzu Corp.). Cell suspension from the fermentor was pumped continuously through a cuvette. The excitation wavelength was 366 nm, and the emission wavelength was 460 nm.

Determination of acetoacetate decarboxylase activity. The activity of acetoacetate decarboxylase was measured mano-

metrically in a Warburg apparatus (1). Cell suspension (10 ml) was centrifuged at 4,000 rpm for 15 min (Christ, Osterode/Harz, Germany). The supernatant was discarded, and the cell pellet was washed once with 1 mM potassium phosphate buffer (pH 7). Afterwards, the cells were suspended in 2 ml of potassium phosphate buffer and disrupted in a French press at 26,000 lb/in^2; 200 μl of the crude extract was analyzed.

Calculations. The specific production rate in continuous culture was calculated by the equation $[(\Delta c/\Delta t) + D \times c_2] \times 1/p$ (micromoles per hour per milligram of protein), where Δc is the product concentration at time 2 minus the product concentration at time 1, Δt is the time difference, c_2 is the product concentration at time 2, p is the protein concentration (milligrams per milliliter), and D is the dilution rate.

Chemicals. Radioactive chemicals were bought from Amersham-Buchler, Braunschweig, Germany. CoA derivatives, methyl viologen, and luciferase from *P. pyralis* (firefly) were obtained from Sigma Chemie, Munich, Germany. All other chemicals were of reagent grade and were bought from Merck, Darmstadt, Germany. Biochemicals and enzymes were obtained from Boehringer if not described otherwise.

RESULTS

pH shift. The growth rate began to decrease and acid production ceased approximately 3 h after the pH control was turned off (Fig. 1a and b). A significant increase in the solvent concentration in the culture could be observed after 10 to 12 h. For a more precise determination of the onset of solvent formation, the specific production rates of butanol and acetone were calculated; the values showed that solvent production had started to increase by 6 h (Fig. 1c). The actual shift to solventogenesis must have taken place at this time. This is supported by the observation that acetoacetate decarboxylase activity started to increase at about 5 h (Fig. 1d). As indicated by the stippled lines in Fig. 1, induction of the solventogenic enzymes occurred between 4.5 and 6 h after the pH control was switched off, when the external pH reached a value of 4.8. In similar experiments, it was shown that acetoacetate decarboxylase mRNA also appears 4 to 6 h before the increase in acetone in the fermentor vessel can be measured (11). Other investigators showed, under comparable conditions, an increase in stress protein synthesis in the first 10 h of the experiment (33).

The ΔpH of 0.76 unit under standard continuous-culture conditions (pH 5.6) increased to 1.49 units during the first 5 to 7 h after the pH control was switched off (Fig. 2a). The total internal butyrate concentration at pH 5.6 was very high, 360 mM. Because of the increase in the ΔpH, it increased by 80 mM and reached 440 mM during the induction phase (Fig. 2b). After 6 h, the butyrate level in the cells declined, whereas acetate, which was present in the cells in much smaller concentrations, remained at about the same concentration for a while and then slowly increased. These differences are reflected in the concentrations of undissociated acids in the cells (Fig. 2c); a maximum of 30 mM butyric acid was found at the time of shift induction in three independent pH shift experiments. Afterwards, the butyric acid concentration went down, whereas the acetic acid concentration slowly increased. This led to a final concentration of 30 mM total undissociated acids during solventogenesis.

In addition, the intracellular concentrations of CoA derivatives, adenine nucleotides, and NAD(P)H were determined in samples withdrawn from the continuous culture after the

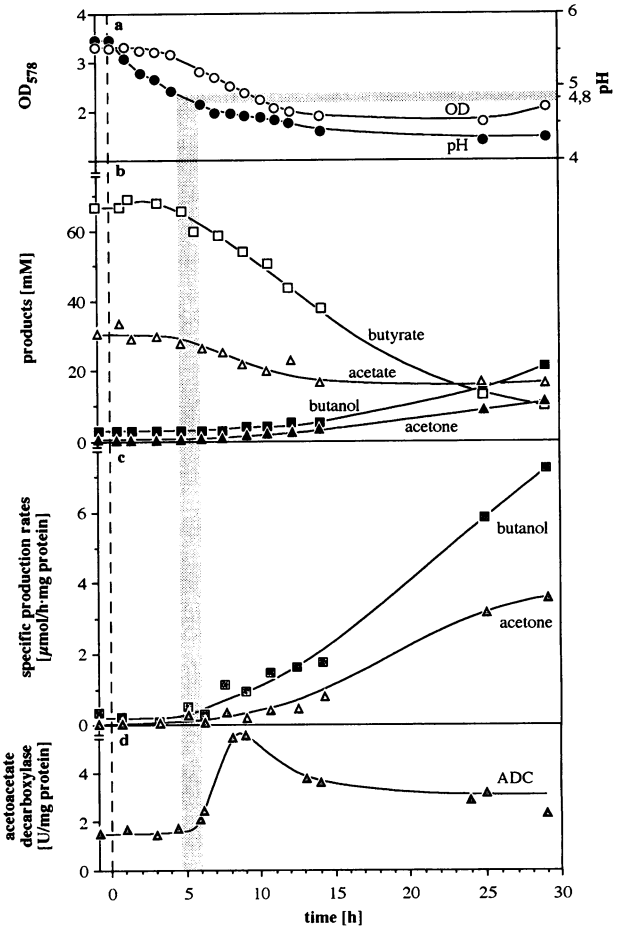

FIG. 1. pH shift experiment in continuous culture. At time zero, the experiment was started by switching off the pH control mechanism. (a) External pH and optical density at 578 nm; (b) fermentation products; (c) specific production rates of solvents; (d) specific activity of the acetoacetate decarboxylase (ADC) (samples were taken from two parallel experiments). Induction occurred after 4.5 to 6 h at an external pH of 4.8, which is indicated by the stippled bar in this and subsequent figures.

cessation of pH control. The results are summarized in Fig. 3. Contrary to our expectations, the intracellular concentrations of butyryl-CoA and acetyl-CoA as well as free CoA first decreased and then increased after shift induction. A CoA derivative that first increased and then decreased in concentration, which could balance the CoA level, could not be detected.

The ATP concentration first decreased but increased again shortly before the phase of shift induction was reached. This is in good agreement with the observations made for batch cultures of *C. acetobutylicum* (36). Two processes with opposite effects could be responsible for this: increasing ATP consumption by the proton-translocating ATPase to maintain the ΔpH under conditions of acid production and increases in the rate of glycolysis.

An interesting time profile was obtained for the concentrations of NADH and NADPH. The content was low under steady-state conditions (0.1 to 0.3 mM) but increased sharply after the pH control had been switched off. It reached a maximum of 1.2 mM during shift induction, decreased again,

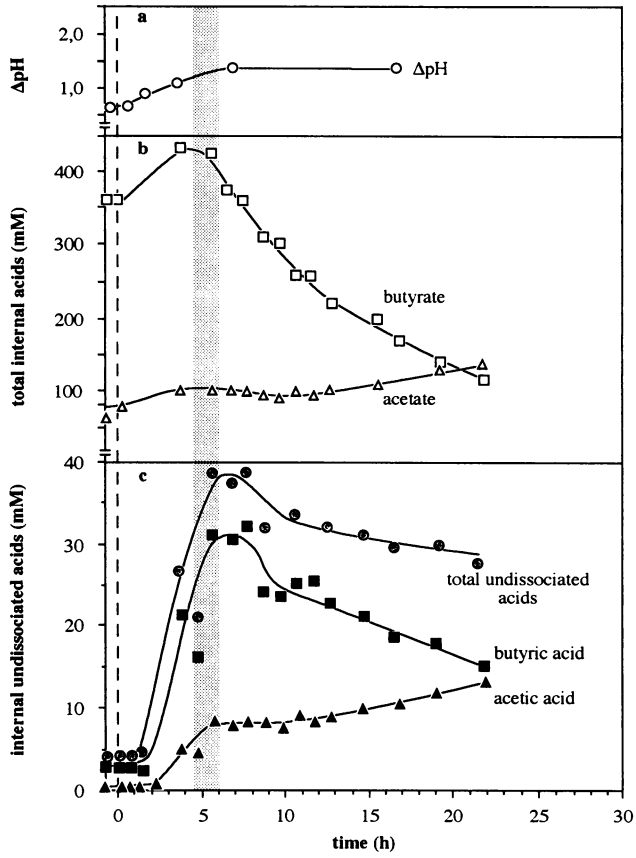

FIG. 2. ΔpH and internal acid concentrations during a pH shift. (a) ΔpH; (b) total butyrate and acetate concentrations; (c) internal undissociated acids. As in Fig. 1, the experiment was started by switching off the pH control at zero.

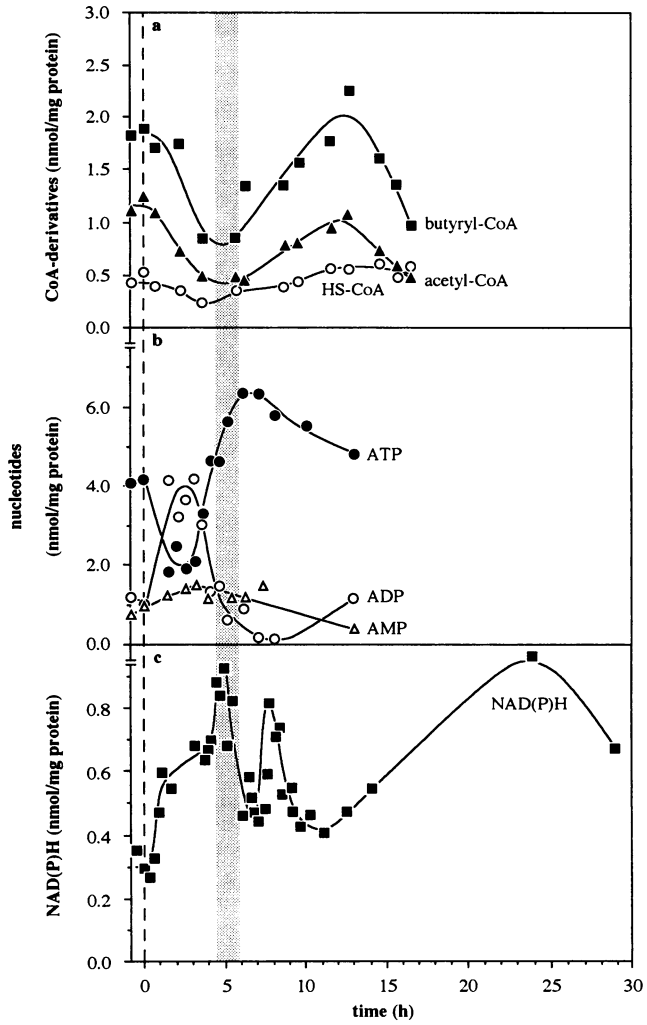

FIG. 3. Time course of some pool concentrations during the pH shift. (a) CoA derivatives; (b) adenine nucleotides; (c) NAD(P)H. The experiment was started by switching off the pH control at time zero. For explanation, see the text.

and then oscillated between 0.4 and 1 mM. These results are in good agreement with those of other investigators, who found increased ATP and NADH levels associated with solvent production (28).

Metabolic oscillation. A net change in the culture redox potential was found to oscillate for at least 80 h, at reproducible intervals of 5 to 7 h, after solvent induction (Fig. 4). Changes in the NAD(P)H and ATP content were seen to parallel the redox oscillation fairly well (Fig. 5). However, no changes in product concentration or hydrogen evolution were observed. The changes for both NAD(P)H and ATP were in the range of 0.5 to 1 mM. Because of its very low level in the cells (0.2 to 1.2 mM), the NAD(P)H pool showed a more pronounced oscillation than that of ATP, which is present at higher concentrations (5 to 6 mM).

Shift induced by addition of acid or methyl viologen. The shift in continuous cultures was also induced by addition of butyrate and acetate at 50 mM each or of methyl viologen to a final concentration of 1 mM. The increases in the specific production rates of butanol are depicted in Fig. 6a and b. It is apparent that the methyl viologen-induced shift occurred almost instantaneously. The acid-induced shift was comparable to the pH-induced shift (compare Fig. 6a and 1c). Acetoacetate decarboxylase activity was indeed induced sooner, but the onset of solvent production was not that much different. In the presence of rifampin, only a transient formation of butanol was observed in the methyl viologen-shifted cultures, indicating that protein synthesis was required.

The time profiles of ATP, NAD(P)H, and acetoacetate decarboxylase levels after the addition of acids or methyl viologen are depicted in Fig. 6c to h. The NAD(P)H and ATP levels increased after the additions, whereas acetoacetate decarboxylase was only induced with acids, not with methyl viologen. In agreement with this, butanol but not acetone was produced following shift induction by methyl viologen. The time course of the ATP levels indicated another difference. During the pH (Fig. 3) as well as the acid shift, ATP levels reached a minimum value of approximately 2 nmol/mg of protein. Such a minimum was not observed with methyl viologen-shifted cells, in which the ATP level stayed at 4 nmol/mg of protein and then increased. The NAD(P)H levels increased more rapidly after the methyl viologen shift than in pH- and acid-shifted cells. Although the final levels of NAD(P)H were equally high under the three conditions, hydrogen evolution was completely inhibited in methyl viologen-shifted cells. This indicates that other redox reactions follow the NAD(P)H increase.

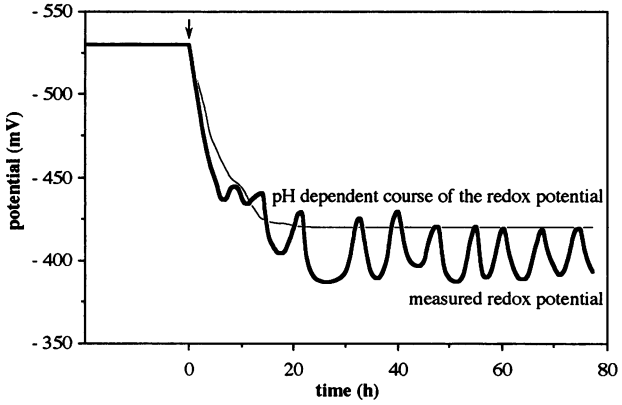

FIG. 4. Oscillation of the redox potential during the pH shift. The arrow mark the time that the pH control was switched off. The pH-dependent course of the redox potential is indicated by the thin line.

DISCUSSION

From the results of other laboratories and the results presented here, it is evident that two sets of shift-inducing conditions can be distinguished (14): (i) lowering the pH or adding various organic acids or uncouplers leads to an increase in the internal concentration of acids, notably butyric acid, and this results in the formation of both acetone and butanol; (ii) the inhibition of H_2 evolution by carbon monoxide, methyl viologen, or iron limitation causes an increased level of NAD(P)H in the cells, and butanol is produced exclusively (e.g., following the methyl viologen shift) or predominantly. Together with the results reported here, this leads to the model for shift induction depicted in Fig. 7.

pH or acid shift. Glycolysis leads to high intracellular acid concentrations, which require increased proton translocation for the maintenance of a ΔpH. This is accomplished by the F_0F_1-ATPase and results in a decrease in the intracellular ATP concentration and a corresponding increase in the ADP concentration. This change in the ATP and ADP ratio is proposed to initiate the generation of signal 1, which leads to induction of the acetoacetate decarboxylase and CoA-transferase. These enzymes accomplish the conversion of acetyl-CoA and acetate to acetone and CO_2:

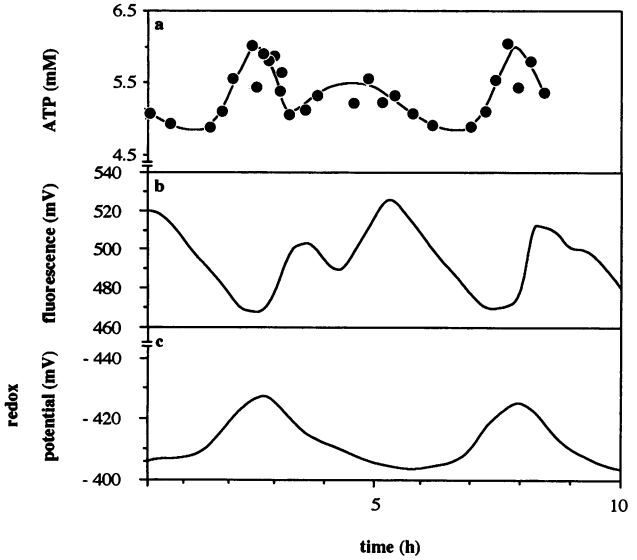

FIG. 5. Oscillation of the ATP pool and culture fluorescence as a measure of the cellular NAD(P)H content. (a) ATP; (b) culture fluorescence; (c) redox potential. The time course of culture fluorescence was compared with parallel determinations of the NAD(P)H concentration by bioluminescence measurements.

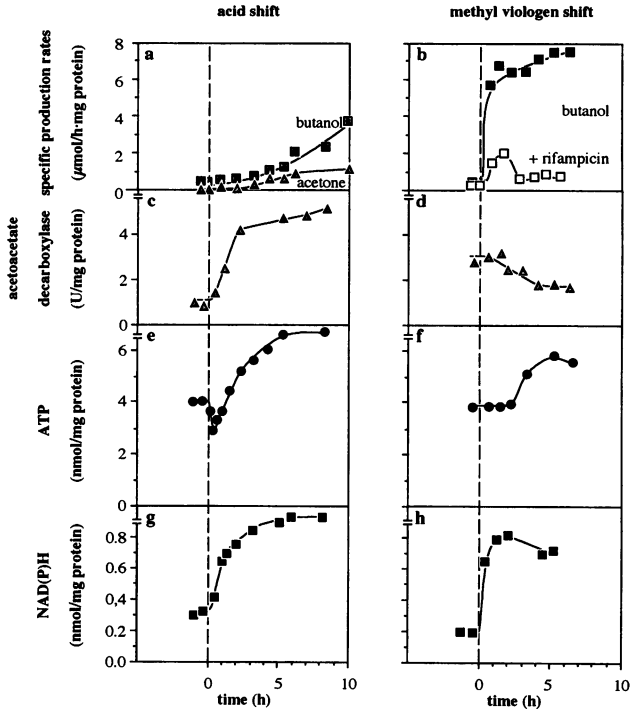

FIG. 6. Comparison of intracellular events during acid- and methyl viologen-induced shifts. (a and b) Specific production rates of solvents; (c and d) specific activity of acetoacetate decarboxylase; (e and f) intracellular ATP concentration; (g and h) cellular NAD(P)H content. The broken lines indicate the time of addition of potassium acetate plus potassium butyrate (acid shift) or methyl viologen.

2 acetyl-CoA	→ acetoacetyl-CoA + CoA
acetoacetyl-CoA + acetate	→ acetoacetate + acetyl-CoA
acetoacetate	→ acetone + CO_2
acetyl-CoA + acetate	→ acetone + CO_2

Thus, net acid is consumed, diminishing the cells' difficulties. In this connection, studies in which fewer phosphorylated proteins were found in cells in the solventogenic phase than in cells in the acidogenic phase should be mentioned (6). Because of acetone formation, the redox balance cannot be stabilized during glycolysis, since adequate amounts of acetyl-CoA are lacking to act as H acceptors, and signal 2, a compound in redox equilibration with NAD(P)H, is generated. The synthesis of butyraldehyde and butanol dehydrogenases is induced, and acetone and butanol formation can proceed. Here, studies in which high levels of rubredoxin were reported to correlate with shift induction and solvent production are of importance (25). The small redox protein rubredoxin is in equilibrium with NADH via an NADH: rubredoxin oxidoreductase, which is positively regulated by low pH and high acetic acid concentration (5, 24, 26, 31, 32).

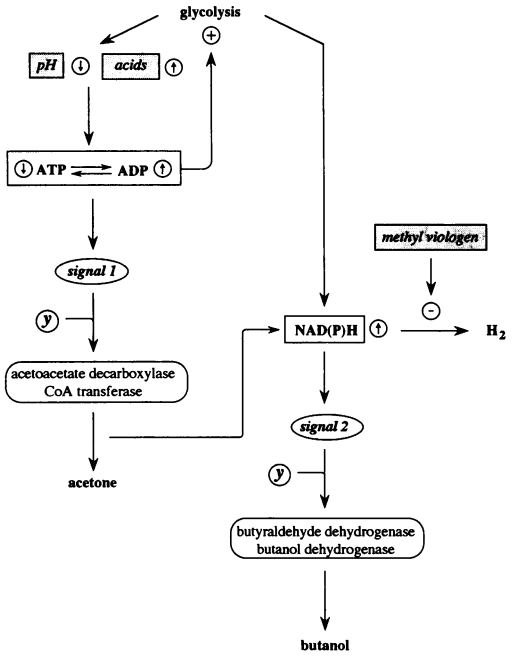

FIG. 7. Model for the shift from acidogenesis to solventogenesis. ⊕, increase; ⊖, decrease in level or activity; ⊕, stimulation; ⊖, inhibition; *y*, common regulatory factor.

Methyl viologen shift. Signal 1 is not generated after a methyl viologen-induced shift, and the enzymes required for acetone formation are not synthesized. Because of the inhibition of hydrogenase, however, signal 2 appears and butanol is produced.

Studies on mutants of *C. acetobutylicum* with a single transposon inserted into the chromosome, which are defective in acetone as well as in butanol formation, indicate a common regulatory factor in solventogenesis (7). These mutants are not able to shift to butanol production after methyl viologen addition (13a).

Metabolic oscillation and signal generation. Oscillation of the culture redox potential and of the intracellular NAD(P)H and ATP concentrations can be observed under certain conditions of solventogenesis in continuous culture. Redox oscillations as well as periodic changes in product concentrations have also been reported by other investigators (9, 21). An explanation can now be offered. The shift to solventogenesis and subsequent growth lead to a recovery in the ATP pool and a decline in the NAD(P)H concentration. Thus, the signals are diluted out and the levels of the solventogenic enzymes decrease. Solvent production declines, acids are produced, and the cells then go through another shift induction cycle. These periodic changes presumably lead to the observed metabolic oscillations.

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